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ORIGINAL ARTICLE

MicroRNA-22 suppresses NLRP3/CASP1 inflammasome pathway-mediated proinflammatory cytokine production by targeting the HIF-1α and NLRP3 in human dental pulp fibroblasts

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Abstract

Aim: To investigate the synergetic regulatory effect of miR-22 on HIF-1α and NLRP3, subsequently regulating the production of the NLRP3/CASP1 inflammasome pathway-mediated proinflammatory cytokines IL-1β and IL-18 in human dental pulp fibroblasts (HDPFs) during the progression of pulpitis.

Methodology: Fluorescence in situ hybridization (FISH) and immunofluorescence (IF) were performed to determine the localization of miR-22-3p, NLRP3 and HIF-1α in human dental pulp tissues (HDPTs). The miR-22 mimics and inhibitor or plasmid of NLRP3 or HIF-1α were used to upregulate or downregulate miR-22 or NLRP3 or HIF-1α in HDPFs, respectively. Computational prediction via TargetScan 5.1 and a luciferase reporter assay were conducted to confirm target association. The mRNA and protein expression of HIF-1α, NLRP3, caspase-1, IL-1β and IL-18 were determined by qRT-PCR and western blotting, respectively. The release of IL-1β and IL-18 was analysed by ELISA. The significance of the differences between the experimental and control groups was determined by one-way analysis of variance, $p < .05$ indicated statistical significance.

Results: A decrease in miR-22 and an increase in HIF-1α and NLRP3 in HDPTs occurred during the transformation of reversible pulpitis into irreversible pulpitis compared with that in the healthy pulp tissues ($p < .05$). In the normal HDPTs, miR-22-3p was extensively expressed in dental pulp cells. HIF-1α and NLRP3 were mainly expressed in the odontoblasts and vascular endothelial cells. Whereas in

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the inflamed HDPTs, the odontoblast layers were disrupted. HDPFs were positive for miR-22-3p, HIF-1α and NLRP3. Computational prediction via TargetScan 5.1 and luciferase reporter assays confirmed that both NLRP3 and HIF-1α were direct targets of miR-22 in HDPFs. The miR-22 inhibitor further promoted the activation of NLRP3/CASP1 inflammasome pathway induced by ATP plus LPS and hypoxia ($p < .05$). In contrast, the miR-22 mimic significantly inhibited the NLRP3/CASP1 inflammasome pathway activation induced by ATP plus LPS and hypoxia ($p < .05$).

Conclusion: MiR-22, as a synergetic negative regulator, is involved in controlling the secretion of proinflammatory cytokines mediated by the NLRP3/CASP1 inflammasome pathway by targeting NLRP3 and HIF-1α. These results provide a novel function and mechanism of miR-22-HIF-1α-NLRP3 signalling in the control of proinflammatory cytokine secretion, thus indicating a potential therapeutic strategy for future endodontic treatment.

KEYWORDS

HIF-1α, human dental pulp fibroblasts, IL-1β, miR-22, NLRP3, pulpitis

INTRODUCTION

Dental caries is one of the most prevalent infectious diseases in the world and results in demineralization of enamel and dentine and subsequent pulpal tissue damage (Cooper et al., 2010). Cellular and molecular responses occur in the pulp in response to dental caries when the infection with caries-related microorganisms reaches the dentinal tubules and pulp connective tissues (Hahn & Liewehr, 2007). During various disease progression, the pulp tissue, like any other injured tissue in the body, initially mounts a defence response including inflammation and regeneration in an attempt to remove the infection and enable tissue healing (Duncan et al., 2019). The two processes of inflammation and regeneration in the pulp tissue appear to be complex and even contradictory; however, a fine balance may exist (Cooper et al., 2014). To defend against invading microbes, cells within pulp tissue release a range of molecular mediators, such as proinflammatory cytokines and chemokines, via innate and/or adaptive immune responses (Khorasani et al., 2020). These proinflammatory cytokines and chemokines play crucial roles in maintaining the balance of inflammation and repair by recruiting inflammatory and immune cells, as well as stem/progenitor cells to the site of infection and injury (Khorasani et al., 2020). Subsequently, these cells attempt to eliminate the invading bacteria, remove any resulting host tissue debris and promote pulp tissue healing and dentine regeneration (Farges et al., 2015).

The NLR family, pyrin domain containing 3 (NLRP3) inflammasome, which is composed of the NLRP3 scaffold, the ASC (PYCARD) adaptor and caspase-1, is a molecular platform that is activated upon cellular infection or stress, triggering the maturation of proinflammatory cytokines, such as IL-1β and IL-18, and engaging innate immune defences (Schroder & Tschopp, 2010). As one of the most powerful proinflammatory cytokines, IL-1β plays a key role in the processes of inflammation and regeneration of pulp tissue. The main function of IL-1β during infection includes not only the rapid recruitment of immune cells, such as neutrophils, to inflammatory sites but also the promotion of the mineralization of dental pulp stem cells (DPSCs) (Khorasani et al., 2020; Yang et al., 2011). A previous study reported that the NLRP3/CASP1 inflammasome pathway played an important role in the development of pulpitis and that the pathway was activated in pulp tissue with irreversible pulpitis (Jiang et al., 2015). However, the mechanism of NLRP3/CASP1 inflammasome pathway activation in irreversible pulpitis remains unclear. Anatomically, the tooth represents a specialized environment with low compliance and a limited tissue swelling capacity and has a relatively poor lymphatic drainage system (Cooper et al., 2014). Oxygen reaches the pulpal cells only through the vasculature in narrow root canals. Inevitably, dental pulp is trapped in ischemic conditions (hypoxia and serum deprivation) when encountering trauma, inflammation, long-term caries injury or chronic pulpitis. It has been demonstrated that hypoxic microenvironment accelerates the release of proinflammatory cytokines, which may be of significance in aggravating inflammatory responses (Huang et al., 2019; Tian et al., 2021). Considered to be a key transcription factor in hypoxia, the hypoxia-inducible factor 1α (HIF-1α) is involved in the progression of inflammation in the dental

pulp (Fujii et al., 2020). However, the relationship between HIF-1α and NLRP3 in regulating the progression of pulpal inflammation is still unclear.

MicroRNAs (miRNAs) are short, endogenously initiated, noncoding RNAs that result in the degradation or translational suppression of a variety of genes by binding with their targets. It has been reported that some miRNAs target the transcripts of multiple genes in the same gene regulatory networks, which is involved in diverse cellular processes, including inflammation, proliferation, differentiation and apoptosis (Acuna et al., 2020; Fang et al., 2019; Hara et al., 2013). Amongst them, miR-22 has been shown to be important in the innate immune system by regulating the production of proinflammatory cytokines (Wan et al., 2016; Yu et al., 2015). Previous studies have reported that both NLRP3 and HIF-1α 3′UTR have the potential binding sites for miR-22, which indicate miR-22 might be involved in the transcription regulation of NLRP3 and HIF-1α (Guo et al., 2021; Yamakuchi et al., 2011). Upregulation of miR-22 decreases the release of inflammatory cytokines IL-1β mediated by NLRP3/CASP1 inflammasome, thus hampering airway inflammation in asthma (Guo et al., 2021). Additionally, overexpression of miR-22 inhibits hypoxia-induced expression of HIF-1α and vascular endothelial growth factor (VEGF), subsequently leading to reduced endothelial cell growth and invasion in colon cancer (Yamakuchi et al., 2011). However, the role of miR-22 in regulating NLRP3/CASP1-mediated pulpal inflammation in hypoxic microenvironment during the pulpitis phase remains unknown. Therefore, this study aimed to investigate the synergetic effect of miR-22 on HIF-1α and NLRP3, subsequently regulating the production of the NLRP3/CASP1 inflammasome pathway-mediated proinflammatory cytokines IL-1β and IL-18 in human dental pulp fibroblasts (HDPFs), in order to provide novel functions and mechanisms of miR-22-HIF-1α-NLRP3 signalling in regulating NLRP3/CASP1 inflammasome pathway-mediated inflammation in HDPFs.

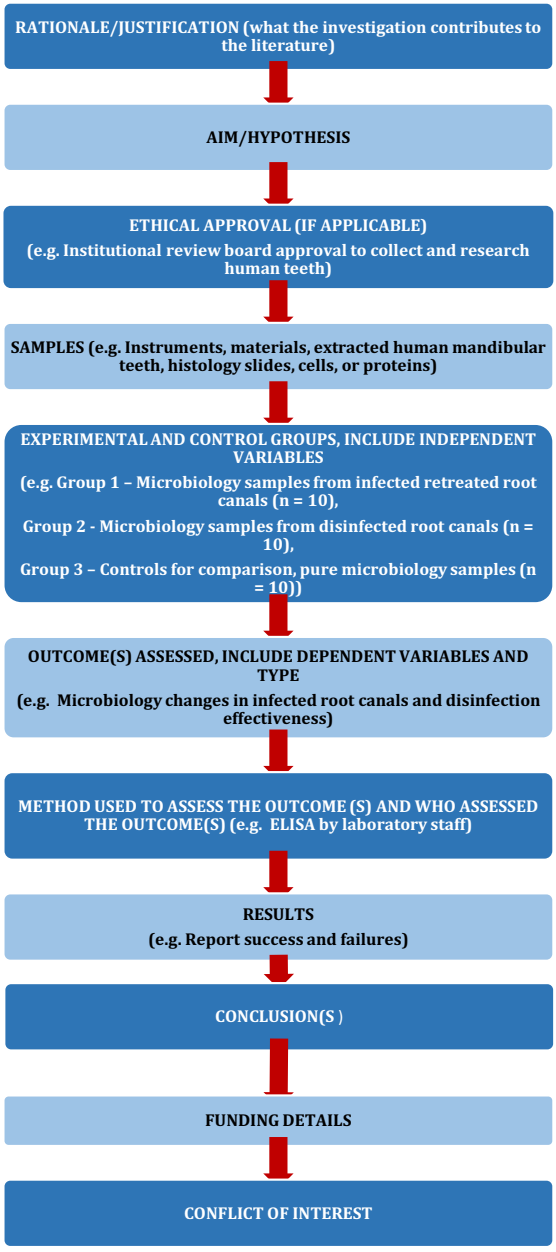

FIGURE 1 PRILE 2021 flowchart (Nagendrababu et al., 2021). For further details visit: http://pride-endodonticguidelines.org/prile

MATERIALS AND METHODS

The manuscript of this laboratory study has been written according to Preferred Reporting Items for Laboratory studies in Endodontology (PRILE) 2021 guidelines (Figure 1) (Nagendrababu et al., 2021).

Sample collection and preparation

The subjects in this study were recruited from the Department of Oral and Maxillofacial Surgery at the School of Stomatology, Fourth Military Medical University, Xi'an, China. Twenty-four human third molars, including eight free from caries, eight carious teeth with reversible pulpitis and eight carious teeth with irreversible pulpitis, were collected for the preparation of human dental pulp tissues (HDPTs), as described previously (Jiang et al., 2015). Clinically, teeth with irreversible pulpitis are sensitive to heat and have spontaneous lingering pain, and teeth with reversible pulpitis show instant pain in cold sensitivity tests. Caries-free teeth from volunteers who had no clinical medical history and took no medications were collected as controls. Clinical and radiographic examinations were used to exclude teeth with a diagnosis of pulp necrosis, periapical pathosis, periodontal diseases or other injuries, except for crown fractures or

any restoration in the normal dental pulp. Written informed consent was obtained from all volunteers and routine surgical procedures were used. The ethics committee of the School of Stomatology, Fourth Military Medical University approved the experimental protocols (permission number IRB-REV-2017-007). The specimens were used for real-time quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR; $n = 5$), fluorescence in situ hybridization (FISH; $n = 3$) and immunofluorescence (IF; $n = 3$). The tooth samples used for FISH and IF were prepared as described previously (Huang et al., 2017; Jiang et al., 2015).

Cell cultures

The pulp tissues were obtained from third molars (donors aged from 17 to 20 years) with the patient's informed consent and ethical approval by the ethics committee of the School of Stomatology, Fourth Military Medical University (permission number IRB-REV-2017-007). All the methods in the study were carried out in accordance with the approved guidelines. HDPFs were cultured and characterized as previously reported (Wang et al., 2021). Briefly, pulp tissues were digested with a 4 mg/ml solution of collagenase/dispase for 1 h at 37°C. Following centrifugation and resuspension in alpha modification of Eagle's medium (α-MEM) supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin, 20% foetal bovine serum (FBS) (Life Technologies). The clonal populations of HDPFs were isolated using a limiting dilution protocol, and cells at the third or fourth passages were used for this study. The normoxic condition referred to a normal oxygen condition, which was maintained with a gas mixture of 21% O_2, 5% CO_2 and 94% N_2. While the hypoxia condition referred to a low oxygen condition, which was maintained with a gas mixture of 1% O_2, 5% CO_2 and 94% N_2 (Fujii et al., 2020). For the ATP plus LPS induction experiments, HDPFs were incubated with ATP (5 mM) for 2 h, and then exposed to LPS (10 μg/ml) for 6 h as previously reported (Jiang et al., 2015).

Reverse-transcriptase PCR and qRT-PCR

Total RNA was extracted from the cells using an RNeasy Mini Kit (QIAGEN) according to the manufacturer's protocol. The total yield of RNA per extraction was calculated using a NanoVue spectrophotometer (GE Healthcare) to measure the absorbance at 260 nm. A260/A280 ratios of 1.9–2.1 indicated extraction of good quality RNA. CDNA was synthesized from 2000 ng RNA using MMLV reverse transcriptase (Promega). Reverse-transcriptase PCR (RT-PCR) and qRT-PCR were performed with RT-PCR kit (Promega) and Precision MasterMix with ROX and SYBR green (PrimerDesign), respectively, as previous reported (Wang et al., 2021). The human gene-specific primers for cDNA can be found in Table 1.

Fluorescence in situ hybridization and immunofluorescence

Fluorescence in situ hybridization of miR-22-3p was performed using a 5′-DIG-and 3′-DIG-labelled miRCURY LNA Detection Probe (Genepharma) and 4-μm tissue sections. The sequence of the miR-22-3p probe was as follows: 5′ Dig-ACAGTTCTTCAACTGGCAGCTT-Dig 3′. The detection of miR-22-3p FISH was performed using FISH Probe Kit (Genepharma) according to the manufacturer's protocol. The procedure of IF was performed as previously described (Jiang et al., 2015). Primary antibodies used for IF were vimentin (1:100; Boster), keratin (1:100; Boster), HIF-1α (1:100; CST), NLRP3 (1:100; CST) at 4°C overnight; PBS was used as the negative control. The secondary antibodies were anti-mouse IgG Alexa Fluor-488 or anti-rabbit IgG Alexa Fluor-594 (1:1000, CST) at room temperature for 1 h. Glass coverslips were mounted using mounting media supplemented with DAPI stain (VectorLabs) and preparations imaged under a fluorescent microscope.

Western blot analysis

The total protein content was extracted from the cells by using lysis buffer containing protease inhibitors (Sigma-Aldrich). The protein concentration was measured by using a BCA-200 protein assay kit (Pierce). Western blot was performed as described previously (Wang et al., 2021). The primary antibodies were HIF-1α (1:500; CST), NLRP3 (1:500; CST), caspase-1 (1:400; CST), IL-1β (1:1000; CST), IL-18 (1:500; CST) and β-actin (1:1000; CST). The secondary antibodies were the horseradish-peroxidase-conjugated anti-mouse IgG antibody or anti-rabbit IgG antibody (1:2000; CST). The activation of caspase-1 was assessed by measuring caspase-1 p20. Protein bands were visualized on X-ray film using an enhanced chemiluminescence system (GE Healthcare). The relative protein expression intensities were quantified by densitometry using Quantity One analysis software (ImageJ software; US National Institutes of Health).

DNA construction and cell transfection

The miR-22 mimic, mimic negative controls (mimic NC), miR-22 inhibitor and inhibitor negative controls

TABLE 1 Primer sequences

Genes	Forward and reverse primers	Accession number
GAPDH	5′-GCACCGTCAAGGCTGAGAAC-3′ 5′-TGGTGAAGACGCCAGTGGA-3′	NM_002046.3
CD146	5′-CATCGTGGCTGTGATTGTG-3′ 5′-TTCTGGGAGCTTATCTGACTTA-3′	NM_006500.3
CD29	5′-AATGTAACCAACCGTAGC-3′ 5′-CAGGTCCATAAGGTAGTAGAG-3′	NM_002211.4
CD90	5′-TAGTGGACCAGAGCCTTCG-3′ 5′-TTCGGGAGCGGTATGTG-3′	NM_006288.3
CD105	5′-CACTAGCCAGGTCTCGAAGGG-3′ 5′-CTGTTTACACTGAGGACCAGAAGC-3′	NM_001114753.2
CD34	5′-GTCTTCCACTCGGTGCGTCTC-3′ 5′-GTTCCCTGGGTAGGTAACTCT-3′	NM_001025109.1
CD45	5′-ATTGCGATTTCCGTGTAA-3′ 5′-CAAGCAGGGCTATTGATG-3′	NM_001267798.2
miR-22	5′-ACACTCCAGCTGGGAAGCTGCCAGTTGAA-3′ 5′-TGGTGTCGTGGAGTCG-3′	NR_029494.1
HIF-1α	5′-GAAACTTCTGGATGCTGGTG-3′ 5′-CAAACTGAGTTAATCCCATG −3′	NM_001243084.1
NLRP3	5′-ATTCGGAGATTGTGGTTGGG-3′ 5′-GAGGGCGTTGTCACTCAGGT-3′	XM_017000183.1
IL-1β	5′-GAATCTCCGACCACCACTAC-3′ 5′-CACATAAGCCTCGTTATCCC-3′	NM_000576.3
IL-18	5′-AGATAGCCAGCCTAGAGGTA-3′ 5′-TTATCAGGAGGATTCATTTC-3′	NM_001243211.2

(inhibitor NC) were obtained from Ribo Biotechnology Company. HDPFs were transfected with the miR-22 mimic (100 nM), mimic NC (100 nM), miR-22 inhibitor (100 nM) and inhibitor NC (100 nM) using Lipofectamine RNAiMAX transfection reagent (Life Technologies). Cells were collected 48 h after transfection. The effects of the miR-22 mimic and miR-22 inhibitor were determined by qRT-PCR.

For NLRP3 or HIF-1α overexpression, the full-length human NLRP3 or HIF-1α coding region was amplified by PCR and cloned into pcDNA3.1 (Invitrogen). Empty vectors (Up NC) were used as NLRP3 or HIF-1α overexpression controls. siRNAs targeting NLRP3 or HIF-1α were obtained from Ribo Biotechnology Company and transfected into cells using Lipofectamine 2000 reagent (Invitrogen). Empty vectors (Down NC, Ribo) were used as NLRP3 or HIF-1α downregulation controls.

Luciferase reporter assay

Wild-type human NLRP3 3′UTR or human HIF-1α 3′UTR luciferase reporter vectors were constructed by amplifying the human NLRP3 mRNA 3′UTR or human HIF-1α 3′UTR and cloning it into the pMIR-REPORT™ Luciferase vector. Constructs with the GCAGC to CGGCG NLRP3 mutation or GGCAG to CCGUC HIF-1α mutation at the putative binding site were also generated and used as controls. HDPFs were cotransfected with 80 ng luciferase reporter plasmid, 40 ng thymidine kinase promoter-Renilla luciferase reporter plasmid and the miR-22 mimic or control (final concentration: 10 nM). After 24 h, luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

ELISA analysis

Upon termination of the treatments, the cell culture supernatants were isolated and stored at −80°C until use. The amount of IL-1β and IL-18 protein in the culture medium was quantified by using a ELISA kit (R&D) following the manufacturer's protocol.

Statistical analysis

Each experiment was performed at least three times unless otherwise indicated. Data are reported as the mean ± SE

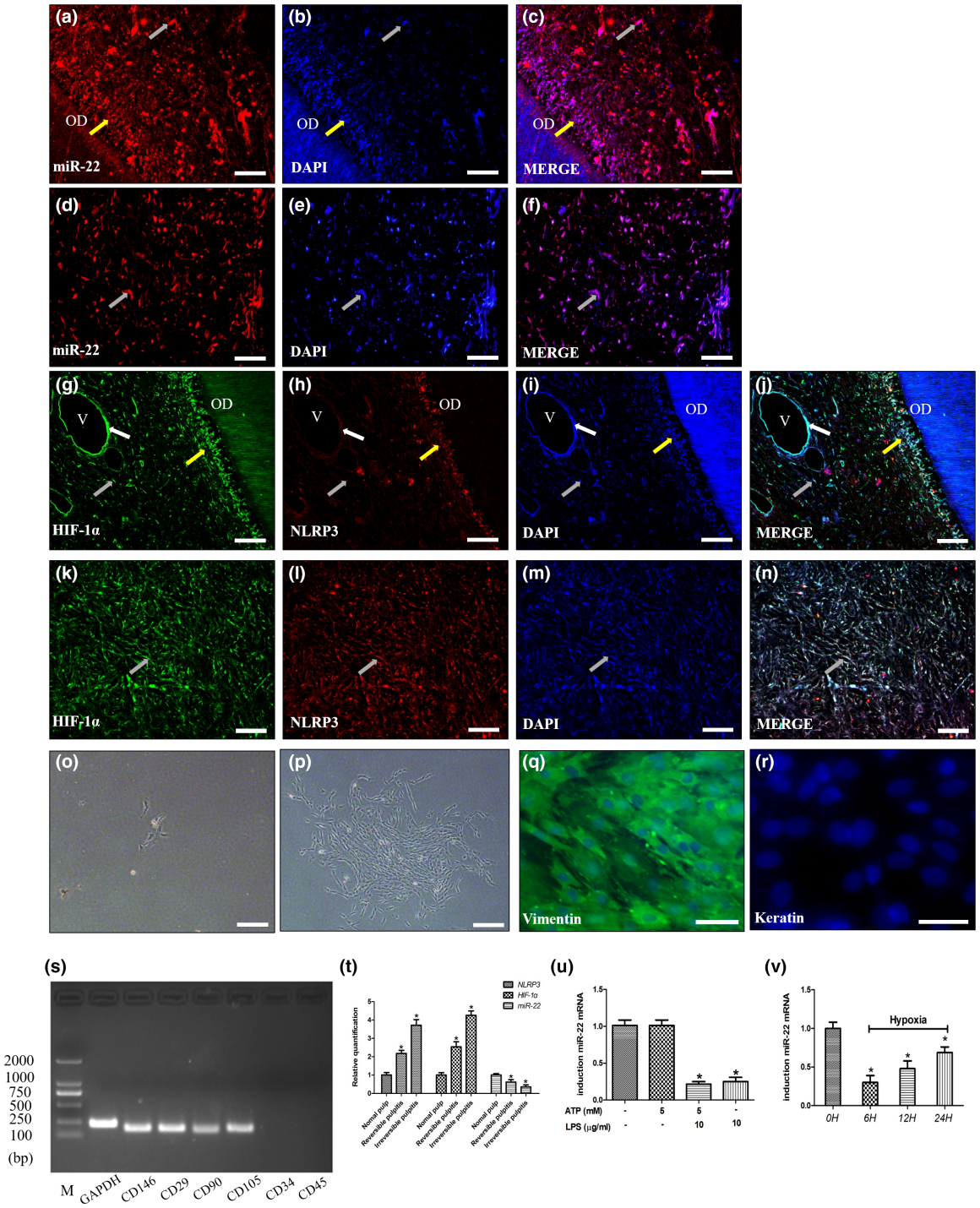

FIGURE 2 The expression of miR-22, NLRP3 and HIF-1α in human dental pulp tissues (HDPTs) and isolation and characterization of human dental pulp fibroblasts (HDPFs). Two groups were stained for miR-22 (red staining) (a–f), HIF-1α (green staining) and NLRP3 (red staining) (g–n): the normal dental pulp tissues and the irreversible pulpitis tissues. Nulei were stained with DAPI (blue staining). Odontoblast (yellow arrows), vascular endothelial cells (white arrows), fibroblasts (grey arrows). The morphological observation of primary cultured HDPFs at day 1 (o) and at day 7 (p). The characterization of HDPFs by immunocytochemical staining and RT-PCR; positive immunostaining for vimentin (q); negative immunostaining for keratin (r); positive for the markers CD146, CD29, CD90, CD105; and negative for the markers CD34 and CD45 (s). Bar: 50 μm. The mRNA expression of miR-22, NLRP3 and HIF-1α was analysed by qRT-PCR (t–v). *$p < .05$ compared with the normal dental pulp tissues or the control group.

(standard error) deviation from three independent experiments. The significance of the differences between the experimental and the control groups was determined by using one-way analysis of variance (ANOVA) with Tukey's Multiple Comparison Test; $p < .05$ indicated statistical significance.

RESULTS

miR-22, NLRP3 and HIF-1α expression levels in HDPTs

Fluorescence in situ hybridization and IF were performed to determine the localization of miR-22-3p, NLRP3 and HIF-1α in the normal pulp and pulp with irreversible pulpitis. In the normal pulp tissues, miR-22-3p was extensively expressed in the dental pulp cells, including odontoblasts and fibroblasts, etc (Figure 2a–c). NLRP3 and HIF-1α were mainly expressed in the odontoblasts and vascular endothelial cells (Figure 2g–j). Some fibroblasts stained positively for HIF-1α, but few fibroblasts stained positively for NLRP3 (Figure 2g–j). In comparison, the dentine and odontoblast layers were disrupted in irreversible pulpitis and dental pulp cells displayed extensive staining for HIF-1α and NLRP3 (Figure 2k–n). The expression of miR-22-3p was downregulated in dental pulp cells in pulp with irreversible pulpitis compared with the normal pulp tissues (Figure 2d–f). Additionally, the mRNA expression of miR-22, NLRP3 and HIF-1α were evaluated in the normal pulp and pulp with reversible or irreversible pulpitis. The mRNA expression of NLRP3 and HIF-1α appeared to increase while the mRNA expression of miR-22 appeared to decrease during the development from reversible to irreversible pulpitis compared to that of the healthy pulp tissue (Figure 2t).

Isolation and characterization of HDPFs

From morphological observation, the primary cells presented clone-like growth (Figure 2o,p). HDPFs obtained from the cell clones were characterized by immunocytochemical staining and RT-PCR. Immunocytochemical staining of HDPFs revealed that the cells positively expressed vimentin (Figure 2q) and were negative for keratin expression (Figure 2r). The RT-PCR results showed that HDPFs expressed a range of mesenchymal cell markers, including CD146, CD29, CD90 and CD105, but not the markers CD34 and CD45 (Figure 2s).

The gene expression of miR-22, NLRP3 and HIF-1α in HDPFs in response to ATP plus LPS or hypoxia

The expression of miR-22 in HDPFs was examined in response to ATP only, LPS only and ATP plus LPS as

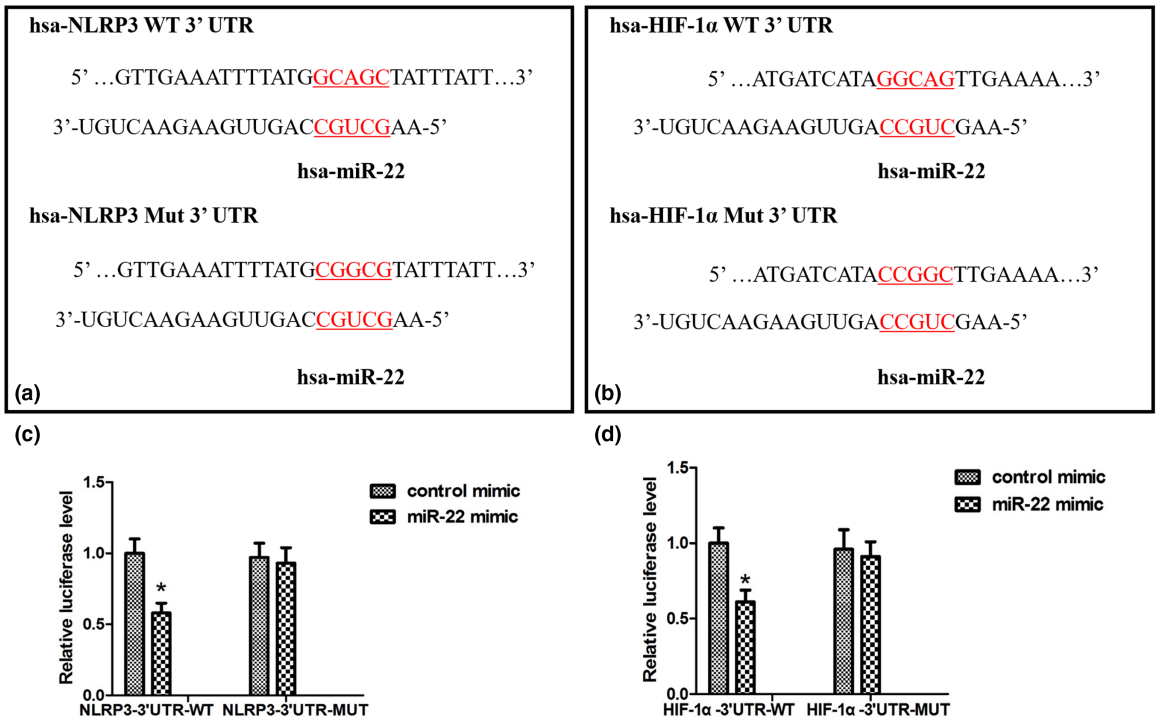

FIGURE 3 miR-22 directly targets NLRP3 and HIF-1α. The targeting site in the 3′UTR of NLRP3 and the corresponding mutant sequence (a). The targeting site in the 3′UTR of HIF-1α and the corresponding mutant sequence (b). Relative luciferase activity of cells after cotransfection with wild-type (WT) or mutant (MUT) NLRP3 3′UTR luciferase reporter vector and the miR-22 mimic or control mimic (c). Relative luciferase activity of cells after cotransfection with wild-type (WT) or mutant (MUT) HIF-1α 3′UTR luciferase reporter vector and the miR-22 mimic or control mimic (d). *$p < .05$ when compared with the miR-con group.

previously described (Jiang et al., 2015). miR-22 decreased significantly in the ATP plus LPS-induced group and LPS-induced group compared to that in the control group (Figure 2u). Additionally, miR-22 expression was evaluated in HDPFs that were cultured in hypoxic conditions for 6 h, 12 h and 24 h. The level of miR-22 decreased notably at 6 h of hypoxia and then increased slightly through 24 h (Figure 2v).

NLRP3 and HIF-1α are miR-22 target genes in HDPFs

Computational prediction via TargetScan 5.1 revealed that miR-22 was one of the non-conserved miRNAs that putatively targets the human 3′UTR of NLRP3 or HIF-1α (Figure 3a,b). The wild-type or mutant 3′UTR of NLRP3 or HIF-1α was cloned into luciferase reporters, and then luciferase reporter assays were performed in HDPFs. Cells were transfected with wild-type or mutant NLRP3-3′UTR luciferase reporter or HIF-1α-3′UTR luciferase reporter plasmid and a miR-22 mimic. The results showed that overexpression of miR-22 markedly decreased luciferase activity compared to that of the control group (Figure 3c,d). In comparison, no change in luciferase activity was observed in cells that were transfected with the mutant NLRP3 3′UTR or mutant HIF-1α 3′UTR construct (Figure 3c,d).

Effects of miR-22 on NLRP3 and subsequent production and secretion of NLRP3/CASP1 inflammasome pathway-mediated proinflammatory cytokines in HDPFs that were induced by ATP plus LPS

Increased and decreased miR-22 levels were observed in HDPFs that were transfected with the miR-22 mimic and miR-22 inhibitor compared to those of the mimic NC and inhibitor NC, respectively (Figure 4a). ATP plus LPS stimulation resulted in the upregulation of NLRP3, IL-1β and IL-18 mRNA (Figure 4b–d) and protein expression compared with those of untreated cells, as well as caspase-1 p20 protein expression (Figure 4g–j) and induced the secretion of IL-1β and IL-18 (Figure 4e,f). In comparison, the miR-22 mimic significantly inhibited ATP plus LPS-induced levels of NLRP3, caspase-1 p20, IL-1β and IL-18, as well as the secretion of IL-1β and IL-18 (Figure 4b–j). The miR-22 mimic decreased the expression of NLRP3, caspase-1 p20, IL-1β and IL-18 induced by ATP plus LPS, which was reversed by upregulating NLRP3 (Figure 5). The miR-22 inhibitor further increased ATP plus LPS-induced expression of NLRP3, caspase-1 p20, IL-1β and IL-18, as well as the secretion of IL-1β and IL-18 (Figure 4b–j). The miR-22 inhibitor increased the expression of NLRP3, caspase-1 p20, IL-1β and IL-18 induced by ATP plus LPS, which was attenuated by downregulating NLRP3 (Figure 6).

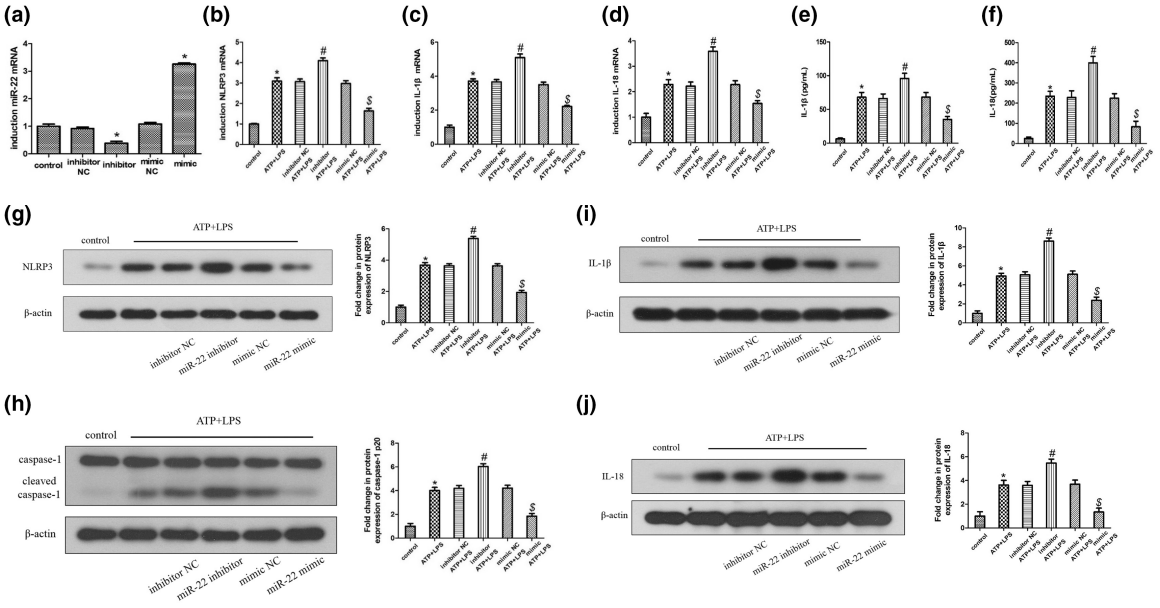

FIGURE 4 miR-22 is a negative regulator of ATP plus LPS-induced NLRP3/CASP1 inflammasome pathway activation in human dental pulp fibroblasts (HDPFs). The mRNA expression of miR-22, NLRP3, IL-1β and IL-18 was analysed by qRT-PCR (a–d). The protein expression of NLRP3, caspase-1, IL-1β, IL-18 and β-actin was analysed by western blotting, and the relative band intensities were determined by densitometry (g–j). The release of IL-1β and IL-18 was analysed by ELISA (e, f). $^{*}p < .05$ compared with the control group. $^{\#}p < .05$ compared with the ATP plus LPS-induced group and inhibitor NC ATP plus LPS-induced group. $^{\$}p < .05$ when compared with the ATP plus LPS-induced group and mimic NC ATP plus LPS-induced group.

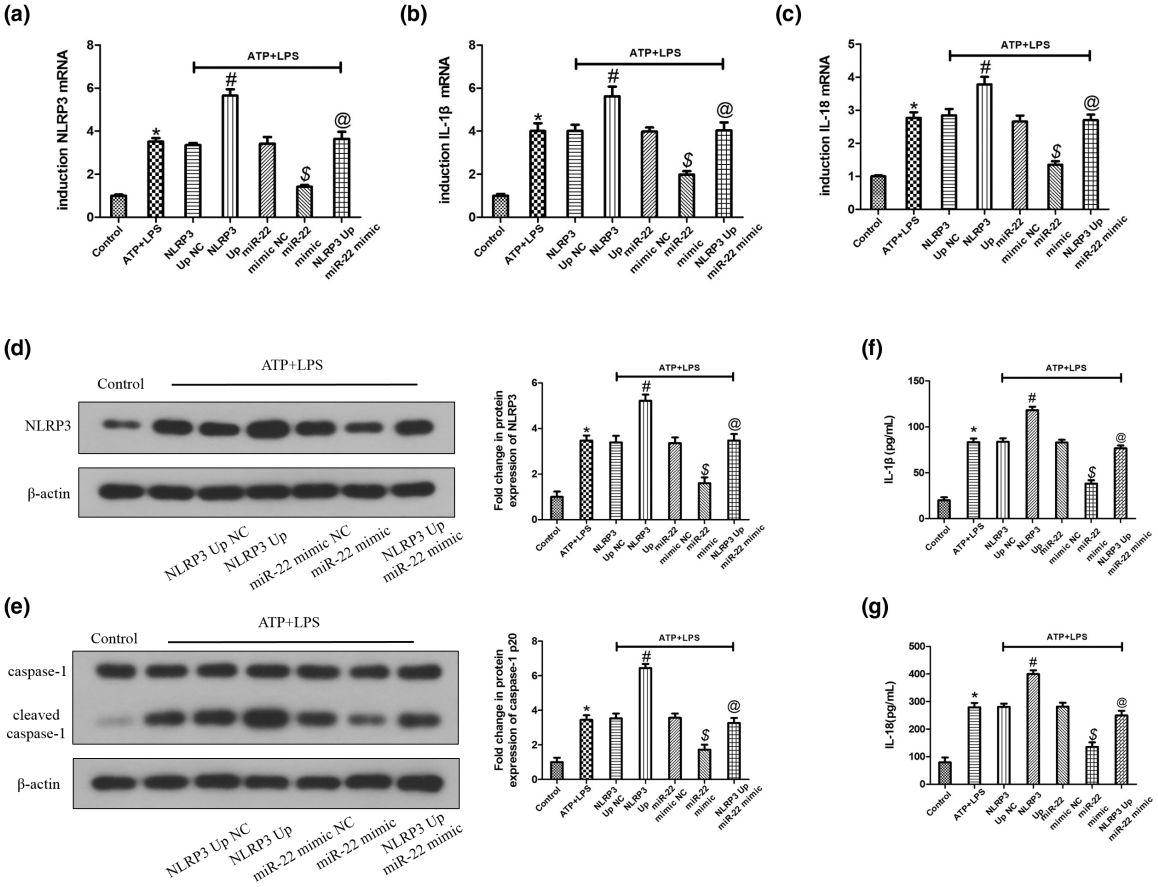

FIGURE 5 Overexpression of miR-22 prohibited the activation of NLRP3/CASP1 inflammasome pathway induced by ATP plus LPS, which was alleviated by upregulating NLRP3 in human dental pulp fibroblasts (HDPFs). The mRNA expression of NLRP3, IL-1β and IL-18 was analysed by qRT-PCR (a–c). The protein expression of NLRP3, caspase-1 and β-actin was analysed by western blotting, and the relative band intensities were determined by densitometry (d, e). The release of IL-1β and IL-18 was analysed by ELISA (f, g). $^{*}p < .05$ compared with the control group. $^{\#}p < .05$ compared with the ATP plus LPS-induced group and NLRP3 Up NC group. $^{\$}p < .05$ compared with the ATP plus LPS-induced group and miR-22 mimic NC group. $^{@}p < .05$ compared with the NLRP3 Up group and miR-22 mimic group.

Effects of miR-22 on HIF-1α and subsequent production and secretion of NLRP3/CASP1 inflammasome pathway-mediated proinflammatory cytokines in HDPFs under hypoxic conditions

Hypoxia resulted in the upregulation of HIF-1α, NLRP3, IL-1β and IL-18 mRNA and protein levels, as well as caspase-1 p20 protein levels (Figure 7a–i), and induced the secretion of IL-1β and IL-18 (Figure 7j,k) compared with those of cells under normoxic conditions. In contrast, the miR-22 mimic significantly inhibited hypoxia-induced the expression of HIF-1α, NLRP3, caspase-1 p20, IL-1β and IL-18 (Figure 7a–i), as well as the secretion of IL-1β and IL-18 (Figure 7j,k). The miR-22 mimic decreased the expression of HIF-1α, NLRP3, caspase-1 p20, IL-1β and IL-18 induced by hypoxia, which was reversed by upregulating HIF-1α in HDPFs (Figure 8). The miR-22 inhibitor further increased hypoxia-induced the levels of HIF-1α, NLRP3, caspase-1 p20, IL-1β and IL-18 (Figure 7a–i), as well as the secretion of IL-1β and IL-18 (Figure 7j,k). The miR-22 inhibitor increased the expression of HIF-1α, NLRP3, caspase-1 p20, IL-1β and IL-18 induced by hypoxia, which was attenuated by downregulating HIF-1α in HDPFs (Figure 9).

miR-22 exerts synergetic effects on HIF-1α and NLRP3 and subsequently regulates the production and secretion of NLRP3/CASP1 inflammasome pathway-mediated proinflammatory cytokines in HDPFs induced by ATP plus LPS and hypoxia

Knockdown of miR-22 further promoted ATP plus LPS- and hypoxia-induced HIF-1α expression (Figure 10a,e), as well as NLRP3/CASP1 inflammasome pathway activation (Figure 10b–d, f–k). Overexpression of miR-22 significantly inhibited HIF-1α expression (Figure 10a,e), as well as NLRP3/CASP1 inflammasome pathway

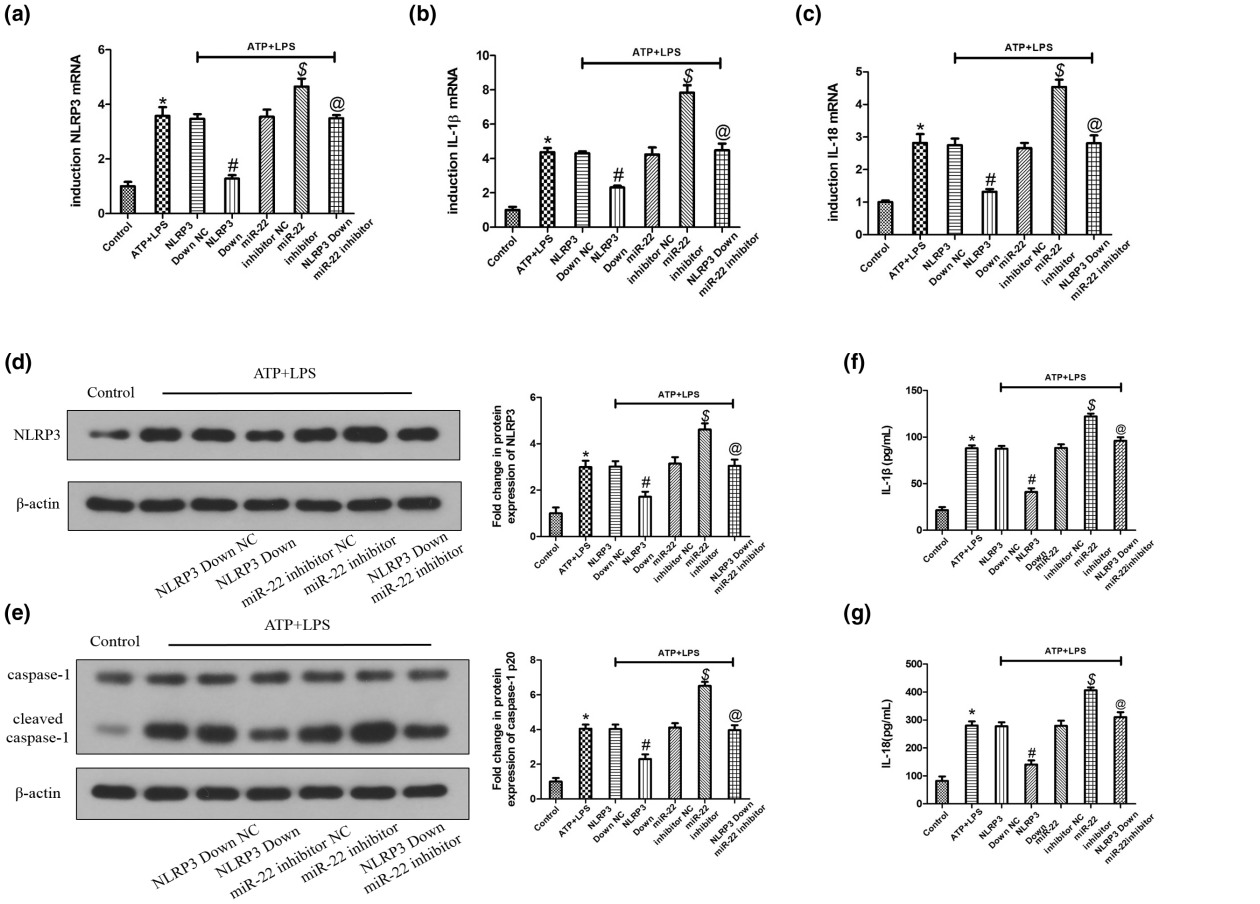

FIGURE 6 Knockdown of miR-22 stimulated the activation of NLRP3/CASP1 inflammasome pathway induced by ATP plus LPS, which was attenuated by downregulating NLRP3 in human dental pulp fibroblasts (HDPFs). The mRNA expression of NLRP3, IL-1β and IL-18 was analysed by qRT-PCR (a–c). The protein expression of NLRP3, caspase-1 and β-actin was analysed by western blotting, and the relative band intensities were determined by densitometry (d, e). The release of IL-1β and IL-18 was analysed by ELISA (f, g). $^{*}p < .05$ compared with the control group. $^{\#}p < .05$ compared with the ATP plus LPS-induced group and NLRP3 Down NC group. $^{\$}p < .05$ compared with the ATP plus LPS-induced group and miR-22 inhibitor NC group. $^{@}p < .05$ compared with the NLRP3 Down group and miR-22 inhibitor group.

activation, in HDPFs induced by ATP plus LPS and hypoxia (Figure 10b–d, f–k).

DISCUSSION

Pulpitis is a typical inflammation that occurs in pulp tissues when infection with caries-related microorganisms reaches the dentinal tubules and pulp. The invasion of these microorganisms subsequently trigger host protective events, including antibacterial, immune and inflammatory responses. Clinically, pulp conditions are categorized into four classes based on the status and symptoms of patients and examinations: normal, reversibly inflamed, irreversibly inflamed and necrotic (Levin et al., 2009; Zheng et al., 2019). Clinical and *in vivo*/*vitro* experimental data clearly indicate that efficient elimination of microorganisms and effective control of pulp immune reactions prevent reversible pulpitis development into irreversible pulpitis and promote dentine barrier formation, thus enabling reestablishment of tissue homeostasis and health (Farges et al., 2015). A variety of cells within pulp tissues detect invading bacteria and subsequently initiate immune responses by expressing a range of pattern recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs) (Cooper et al., 2017). These cells include not only immune cells (such as macrophages and dendritic cells) but also non-immune cells (such as odontoblasts and fibroblasts) (Cooper et al., 2017). HDPFs, the main cells that constitute the dental pulp, play a crucial role in the dental pulpal inflammatory response and immune response when stimulated by caries-related bacterial virulence factors (Jiang et al., 2015). A previous study showed that HDPFs contained increased functional NLRP3/CASP1 inflammasomes in inflamed pulp tissues and that the NLRP3/CASP1 inflammasome pathway was activated in irreversible pulpitis *in vivo*. Additionally, this pathway

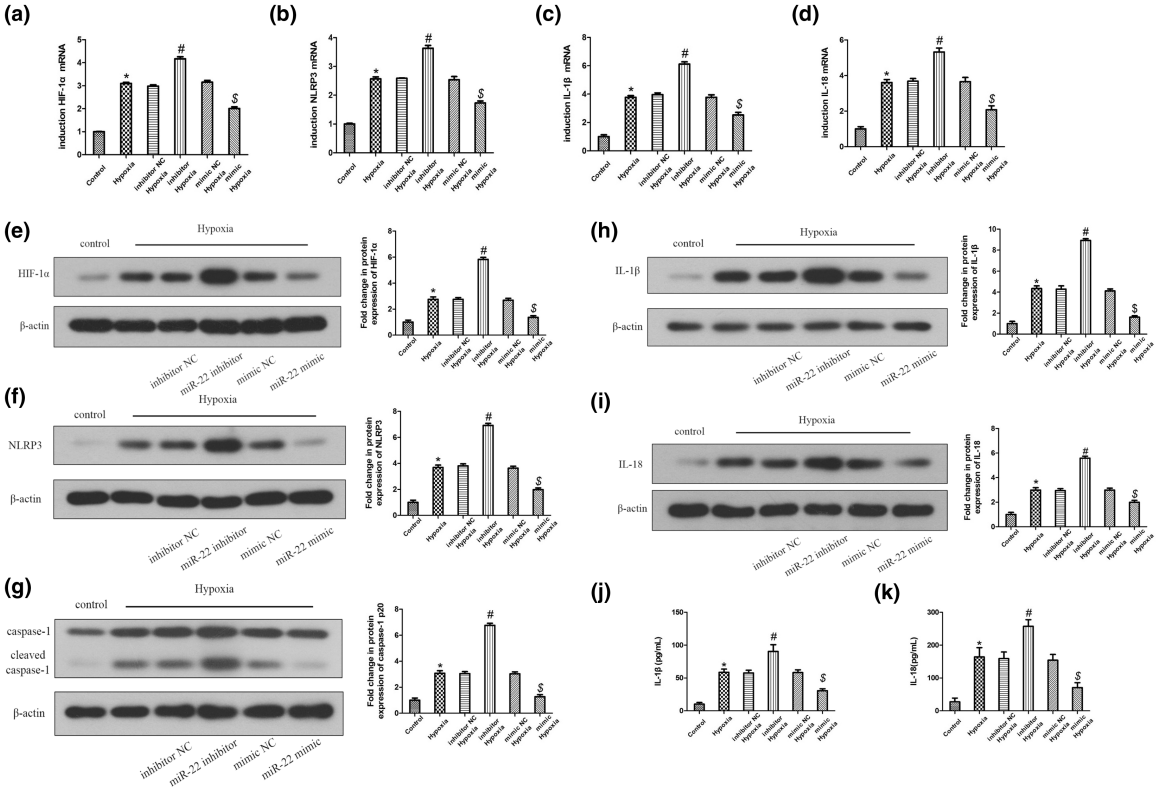

FIGURE 7 miR-22 is a negative regulator of hypoxia-induced NLRP3/CASP1 inflammasome pathway activation in human dental pulp fibroblasts (HDPFs). The mRNA expression of HIF-1α, NLRP3, IL-1β and IL-18 was analysed by qRT-PCR (a–d). The protein expression of HIF-1α, NLRP3, caspase-1, IL-1β, IL-18 and β-actin was analysed by western blotting, and the relative band intensities were determined by densitometry (e–i). The release of IL-1β and IL-18 was analysed by ELISA (j, k). $^{*}p < .05$ compared with the control group. $^{\#}p < .05$ when compared with the hypoxia-induced group and inhibitor NC hypoxia-induced group. $^{\$}p < .05$ compared with the hypoxia-induced group and mimic NC hypoxia-induced group.

was activated by LPS through a process involving the ATP-activated $P2X_7$ receptor ATP-gated ion channel in HDPFs *in vitro* (Jiang et al., 2015). In this study, upregulation of NLRP3 and downregulation of miR-22 were demonstrated in the progression of reversible pulpitis into irreversible pulpitis *in vivo*. ATP plus LPS induction reduced miR-22 expression in HDPFs *ex vivo*. A large number of studies have reported that miRNAs have gained importance as gene expression regulators in the immune responses against various pathogens by regulating the production of inflammatory mediators (Chandan et al., 2019). Increasing evidence demonstrates that several miRNAs directly target a range of PRRs to modulate immune processes (Zhou et al., 2018). Teng et al. (2013) reported that Let-7b regulated the activation of NF-κB and its downstream genes related to inflammation and immune responses by targeting toll-like receptor (TLR)-4 in gastric epithelium induced by *H. pylori* infection. In addition, Ma et al. (2014) showed that miR-124 exerted a negative effect on the inflammatory response in alveolar macrophages upon mycobacterial infection by directly targeting TLR-6. Herein, computational prediction via TargetScan 5.1 and luciferase reporter assays confirmed that the 3′UTR of NLRP3 was a target site of miR-22 in HDPFs. Experiments using a miR-22 mimic and inhibitor revealed that miR-22 regulated the NLRP3/CASP1 inflammasome pathway in HDPFs in response to ATP plus LPS. Taken together, we found that miR-22 acted as a negative regulator of NLRP3/CASP1 inflammasome pathway activation by directly targeting NLRP3.

Due to the encasement of pulp tissue within rigid dentine walls, dental pulp is trapped in low oxygen conditions and low glucose conditions when circulatory impairment is induced by the initiation of inflammation (Yu et al., 2002). *In vivo* study, immunofluorescence staining showed that HIF-1α and NLRP3 were mainly present in the odontoblast layer and vascular endothelial cells of the normal pulp tissues. However, in the pulp tissues showing irreversible pulpitis, the infection of caries-related microorganisms destroyed the odontoblast layers and reached pulp tissue. The HIF-1α and NLRP3 proteins had been shown to be widely expressed in the dental pulp cells, especially in fibroblasts. Additionally, the gene expression of HIF-1α was upregulated during the progression of

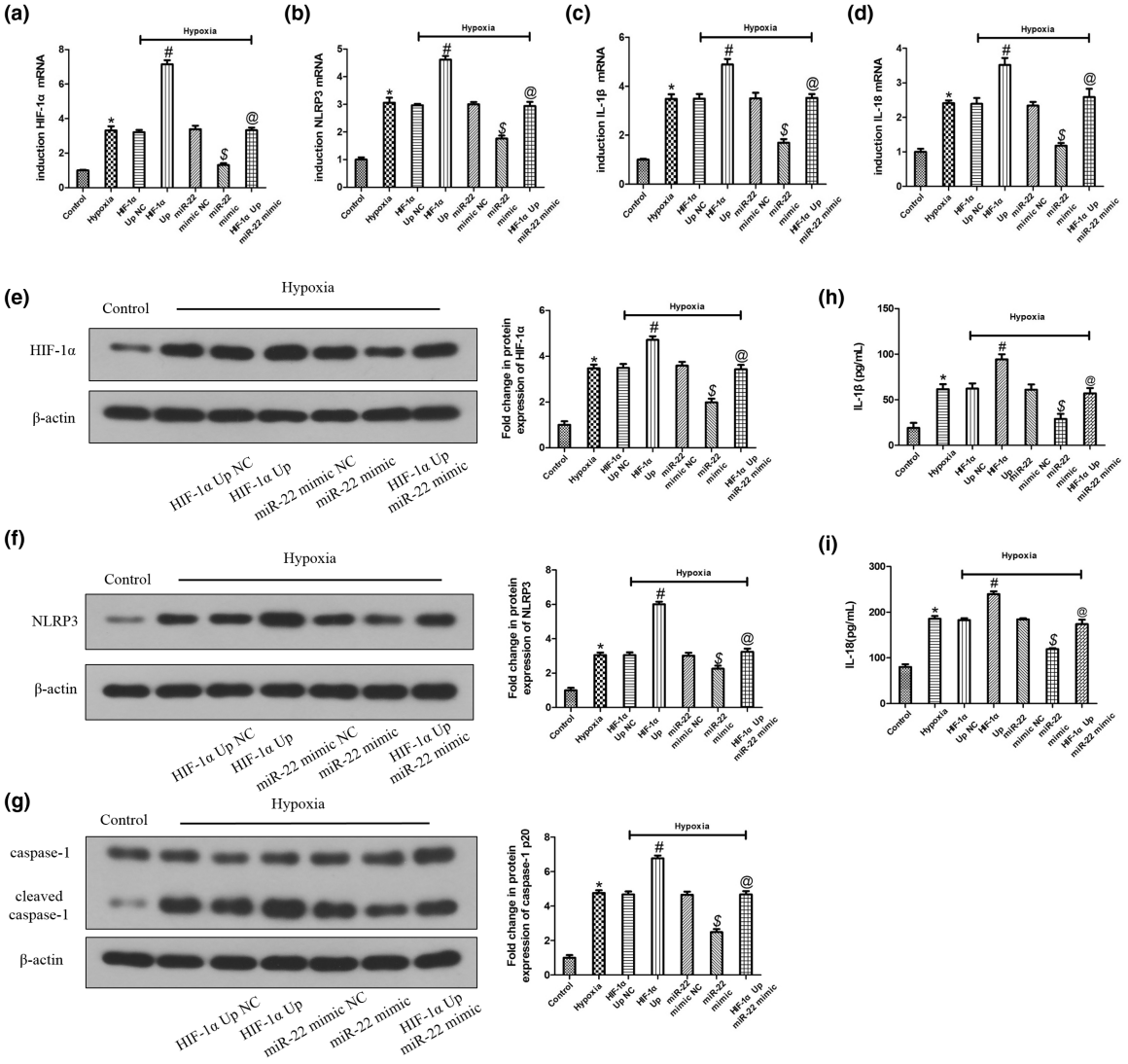

FIGURE 8 Overexpression of miR-22 prohibited the activation of NLRP3/CASP1 inflammasome pathway induced by hypoxia, which was alleviated by upregulating HIF-1α in human dental pulp fibroblasts (HDPFs). The mRNA expression of HIF-1α, NLRP3, IL-1β and IL-18 was analysed by qRT-PCR (a–d). The protein expression of HIF-1α, NLRP3, caspase-1 and β-actin was analysed by western blotting, and the relative band intensities were determined by densitometry (e–g). The release of IL-1β and IL-18 was analysed by ELISA (h, i). $^{*}p < .05$ compared with the control group. $^{\#}p < .05$ compared with the hypoxia-induced group and HIF-1α Up NC group. $^{\$}p < .05$ compared with the hypoxia-induced group and miR-22 mimic NC group. $^{@}p < .05$ compared with the HIF-1α Up group and miR-22 mimic group.

pulpitis. *In vitro* study, we revealed that miR-22 expression was downregulated when HDPFs were cultured in hypoxic conditions. Computational prediction via TargetScan 5.1 and luciferase reporter assays confirmed that HIF-1α was a direct target of miR-22 in HDPFs. Experiments using a miR-22 mimic and inhibitor revealed that miR-22 regulated the NLRP3/CASP1 inflammasome pathway by targeting HIF-1α in HDPFs in response to the hypoxic conditions. Increasing evidence has demonstrated that some miRNAs are predicted to have hundreds of targets, which illustrates the importance of these miRNAs in various tissues at different stages of development by regulating different targets (Winter et al., 2009). For instance, Chen et al. (2013) reported that miR-21 was involved in hepatitis C virus (HCV)-induced type I interferon (IFN) production by targeting two important factors in the TLR signalling pathway, myeloid differentiation factor 88 (MyD88) and interleukin-1 receptor-associated kinase 1 (IRAK1), in human hepatocytes. In this study, we provided evidence that miR-22 directly targeted both NLRP3 and HIF-1α in HDPFs. We further investigated the role of miR-22 in regulating the activation of NLRP3/CASP1 inflammasome pathway in HDPFs in response to ATP plus LPS, as well as hypoxia. It demonstrated that miR-22 functioned as a synergetic negative regulator, targeting NLRP3 and HIF-1α in ATP plus LPS-stimulated HDPFs that were cultured in hypoxic conditions, to activate the NLRP3/CASP1

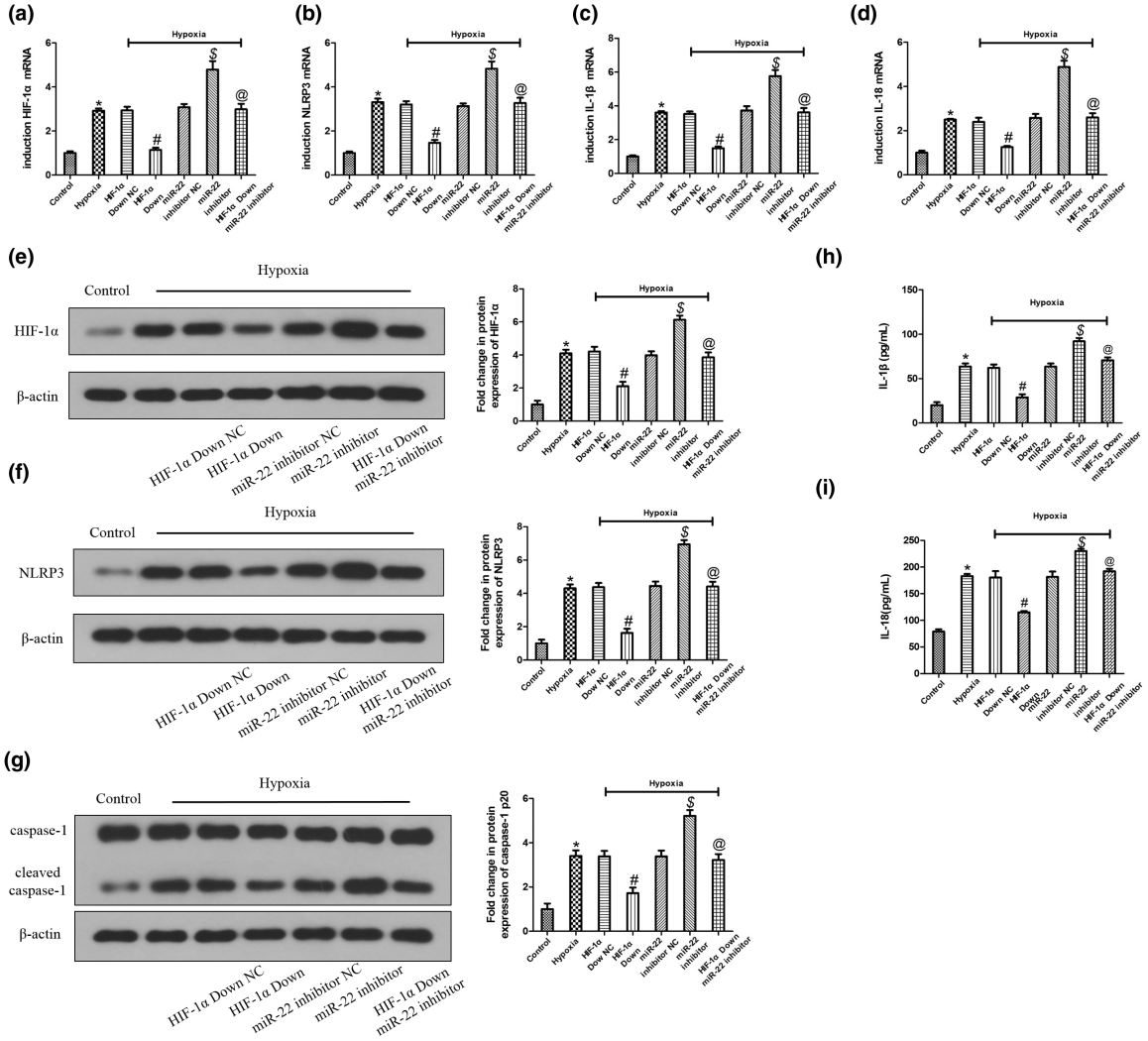

FIGURE 9 Knockdown of miR-22 stimulated the activation of NLRP3/CASP1 inflammasome pathway induced by hypoxia, which was attenuated by downregulaing HIF-1α in human dental pulp fibroblasts (HDPFs). The mRNA expression of HIF-1α, NLRP3, IL-1β and IL-18 was analysed by qRT-PCR (a–d). The protein expression of HIF-1α, NLRP3, caspase-1 and β-actin was analysed by western blotting, and the relative band intensities were determined by densitometry (e–g). The release of IL-1β and IL-18 was analysed by ELISA (h, i). $^{*}p < .05$ compared with the control group. $^{\#}p < .05$ compared with the hypoxia-induced group and HIF-1α Down NC group. $^{\$}p < .05$ compared with the hypoxia-induced group and miR-22 inhibitor NC group. $^{@}p < .05$ compared with the HIF-1α Down group and miR-22 inhibitor group.

inflammasome pathway, thereby promoting pulpal innate immune responses.

Cytokines and chemokines are small molecule immune mediators within the dental pulp that play important roles in inflammatory responses, immune responses and regenerative responses of dental pulp (Khorasani et al., 2020). Produced and secreted by immune and non-immune cells in the pulp tissue, these proteins affect many interactions between these cells, including proliferation, migration, inflammation, differentiation, apoptosis and many others. As a predominant cytokine in inflammation of the dental pulp, IL-1β plays a crucial role in dental pulp inflammation and regeneration (Yang et al., 2018). An *in vitro* study showed that IL-1β induced human dental pulp cells (HDPCs) to secrete monocyte chemotactic factor-1 (MCP-1), which regulated intercellular adhesion molecule (ICAM) expression in the endothelium and promoted the migration of neutrophils through the capillary wall to reach the inflammatory area (Chang et al., 2016). Neutrophils help trap invading bacteria and prevent their advance in pulp tissue, as well as kill them by phagocytosis (Gautam et al., 2017; Holder et al., 2019). Additionally, findings by Chang et al. (2012) demonstrated that IL-1β played a part in inflammatory responses in pulpitis through stimulation of soluble vascular cell adhesion molecule (sVCAM-1) from HDPCs, thus leading to the recruitment, extravasation and migration of leukocytes. Regarding the role of IL-1β in the regenerative processes of pulp tissue, Nakanishi et al. (2015) reported that IL-1β stimulated HDPCs to produce cyclooxygenase-2 (COX-2),

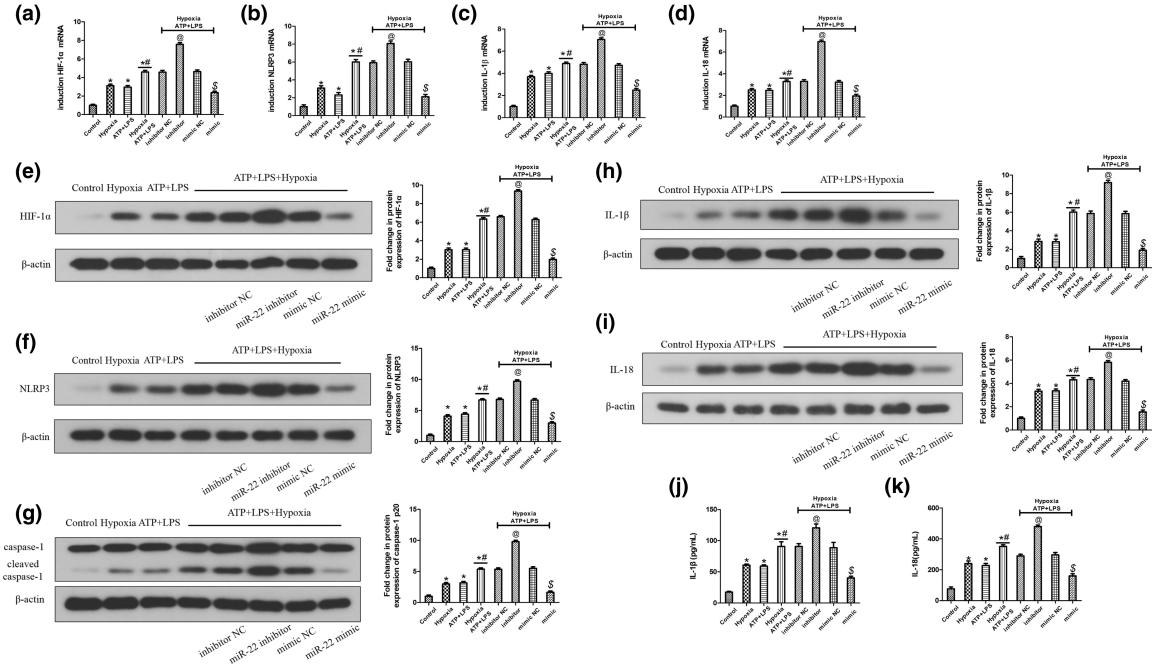

FIGURE 10 miR-22 negatively regulates hypoxia and ATP plus LPS-induced NLRP3/CASP1 inflammasome pathway activation in human dental pulp fibroblasts (HDPFs). The mRNA expression of HIF-1α, NLRP3, IL-1β and IL-18 was analysed by qRT-PCR (a–d). The protein expression of HIF-1α, NLRP3, caspase-1, IL-1β, IL-18 and β-actin was analysed by western blotting, and the relative band intensities were determined by densitometry (e–i). The release of IL-1β and IL-18 was analysed by ELISA (j, k). $^{*}p < .05$ compared with the control group. $^{\#}p < .05$ compared with the hypoxia-induced group and ATP plus LPS-induced group. $^{@}p < .05$ compared with the hypoxia and ATP plus LPS-induced group and inhibitor NC hypoxia and ATP plus LPS-induced group. $^{\$}p < .05$ compared with the hypoxia and ATP plus LPS-induced group and mimic NC hypoxia and ATP plus LPS-induced group.

which induced the expression of VEGF and thus angiogenesis. Furthermore, Ozeki et al. (2014) demonstrated that IL-1β induced the release of matrix metalloproteinase-3 (MMP-3) in HDPCs, which accelerated wound healing following dental pulp injury. Based on the findings of the latest studies, proinflammatory cytokines, such as IL-1β, play important roles in the initiation of immune responses and tissue regeneration in pulp tissue following bacterial infection. However, the production and secretion of these immune mediators act as a double-edged sword. On the one hand, proinflammatory cytokines cause inflammation and tissue damage to the dental pulp; on the other hand, they promote migration of immune cells to eliminate the infectious agent, as well as migration, proliferation and differentiation of stem cells to regenerate tissues. Therefore, controlling the production and secretion of proinflammatory cytokines during the dental pulpal inflammatory phase is of vital importance. In the present study, we demonstrated that miR-22 functioned as an efficient regulator to control the production and secretion of the proinflammatory cytokines IL-1β and IL-18, which were mediated by the NLRP3/CASP1 inflammasome pathway, by targeting NLRP3 and HIF-1α in LPS-stimulated HDPFs under hypoxic conditions. Overexpression of NLRP3 or HIF-1α amplified the secretion of IL-1β and IL-18, which could be reversed by upregulation of miR-22. Knockdown of NLRP3 or HIF-1α decreased the secretion of IL-1β and IL-18, which could be restored by downregulation of miR-22. Our findings contribute to expanding the knowledge of immuno-miRs in host–bacterial infection interactions. Although miRNA-based therapy has limitations, it is a futuristic approach for the diagnosis and treatment of immune-related diseases (acute and chronic inflammatory disorders) and infectious diseases.

CONCLUSIONS

This study demonstrated the upregulation of NLRP3 and HIF-1α, as well as downregulation of miR-22 during the transformation of reversible pulpitis into irreversible pulpitis *in vivo*. Additionally, miR-22, as a synergetic negative regulator, controlled the production and secretion of NLRP3/CASP1 inflammasome pathway-mediated proinflammatory cytokines by targeting NLRP3 and HIF-1α. This study represents a significant advance in better understanding the regulatory effects of immuno-miRs on dental pulpal inflammatory responses.

The findings of novel functions and mechanisms of miR-22-HIF-1α-NLRP3 signalling provide a promising therapeutic strategy for future endodontic treatment.

AUTHOR CONTRIBUTIONS

WJ, SS and DW conducted the investigation, collected and analysed the data. WJ wrote the original draft. JQ, YS, QZ and YZ provided the assistance to the study. WH, BS, WJ and SW reviewed and edited the manuscript. WJ and SW were the main supervisors and initiators of the study. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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